Antioxidants as Neuroprotective Buffer Against Stroke Damage

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with research* in the undergraduate college of The Ohio State University

By

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May 2010

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Abstract

Cerebral ischemia leads to neurological damage and cognitive defects. Restoring blood flow to the brain is the most effective way to limit damage of cerebral tissue; however, reperfusion is also associated with tissue injury in the form of oxidative stress. Oxidative stress is characterized by the generation of free radicals resulting in damage to lipids, proteins and nucleic acids, which in turn contribute to cell death. The antioxidant glutathione is constitutively present in the body and is responsible for neutralizing free radicals produced by oxidative stress. The primary hypothesis of this experiment was that the administration of N-acetyl-L-cysteine (NAC), a glutathione precursor, would act as a buffer against stroke damage. Transient focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO). All experimental animals were treated with either NAC (150mg/kg) or isotonic saline solution. Our behavioral data indicate that MCAO decreases exploratory behavior and induces sensorimotor deficits including reduced mobility and precision of limb control during walking. The treatment of MCAO mice with NAC, however, improved control and strength in the affected hind limb. Additionally, following stroke, NAC-treated mice showed improved mobility in the affected hind limb. Additionally, NAC treatment produced a trend of reduced oxidative stress as well as reduced infarct volume relative to saline treatment, which likely contributed to improved sensorimotor recovery. Taken together, these preliminary data suggest a role for anti-oxidant therapy in improving post-stroke functional recovery.

Introduction

Stroke is the third leading cause of death in the United States and in most industrialized countries after cardiovascular disease and cancer[1, 8]. A stroke is characterized by a rapid onset of neurological impairment due to a block of blood flow to the brain, frequently caused by an arterial thrombus or an embolus lodged from a distant site.[1] When cerebral blood flow falls below a critical

threshold, as it does in ischemic stroke, then the microvascular delivery of vital nutrients to neurons, thus neuronal functioning, is compromised.[4] Currently, tissue plasminogen activator, a thrombolytic agent with a narrow 3-hour therapeutic window, is the only US Food and Drug Administration-approved therapy available for treatment of stroke patients.[6] Lack of other available treatment options has promoted studies to examine the underlying mechanisms of cerebral ischemic injury in order to reveal potential treatments. Studies in humans have discovered that social isolation can have a negative impact on stroke outcome, whereas social support can have neuroprotective effects from the consequences of stroke[3]. Furthermore, affiliative social interaction improves cerebral ischemia outcome.[5] Additional evidence has demonstrated that oxytocin (OT), a neuropeptide released during social interaction, confers neuroprotection by decreasing inflammation and increasing antioxidant activity. In particular, the increased antioxidant activity is believed to reduce stroke damage by combating a condition called oxidative stress. Cerebral ischemia causes a sustained oxidative stress that significantly increases during reperfusion[8]. Oxidative stress following stroke is characterized by the generation of free radicals, specifically reactive oxygen species (ROS) [1], resulting in damage to lipids, proteins and nucleic acids thus contributing to the progression of cell death[10]. The central nervous system is particularly vulnerable to the effects of oxidative damage due to the metabolically expensive character of neurons, the large amount of lipids in the brain being a target for lipid peroxidation and the lack of sufficient antioxidant defenses against ROS[8].

Following stroke, the generation of ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and peroxinitrite ($ONOO^-$) result in lipid peroxidation, excitotoxicity and inflammation[11]. Antioxidants counter the toxic effects of these free radicals by catalyzing their decomposition into less harmful molecules. For example, the enzyme glutathione peroxidase (GPx) catalyzes the reaction between the antioxidant, glutathione, and the ROS, H_2O_2, to water and the oxidized form of glutathione. Following this reaction, the oxidized form of glutathione is recycled back to its

reduced form by the enzyme glutathione reductase. The reduced form of the antioxidant is necessary in order to continue the decomposition of ROS into less harmful molecules.

The neurodegeneration caused by stroke can lead to many forms of impairment including gait impairment. Gait not only is an indicator of lower extremity motor function, but also indicates cognitive function, particularly executive function.[12] Furthermore, retraining of walking is a major goal in the rehabilitation program for persons after stroke.[9] Analyzing gait and ambulation in laboratory animals following MCAO may indicate the extent of the damage caused by oxidative stress. N-acetylcysteine (NAC) has been reported to effectively scavenge ROS and to increase the rate of endogenous glutathione synthesis. Because the antioxidant glutathione eliminates the ROS responsible for causing neuronal damage, its precursor NAC may be a promising therapeutic agent for the treatment of the stroke and its subsequent impairments[8].

The goals of the present study were to determine the effects of post-ischemic antioxidant administration on stroke outcome. The primary purpose of this study was to determine whether treatment with the glutathione precursor, NAC, reduces the oxidative stress, improves mobility, and decreases infarct size following middle cerebral artery occlusion (MCAO). The primary hypothesis was that administration of the antioxidant NAC following the ischemic event improves stroke outcome by decreasing oxidative stress, size of the infarct and gait impairment.

Materials and Methods

Animals

Adult male C57/BL6 mice (Charles River, Wilmington, Massachusetts) weighing 23g to 30g, were maintained on a 14:10 light/dark cycle in a temperature and humidity-controlled vivarium. All animals were allowed *ad libitum* access to food (Harlan Teklad 8640, Indianapolis, IN) and filtered

tap water throughout the study. All experimental animals were housed individually beginning one week prior to surgery and throughout the reperfusion period. The study was conducted in accordance with National Institutes of Health guidelines for the care and use of animals and under protocols approved by The Ohio State University Institutional Animal Care and Use Committee.

Experimental Protocols

Study 1: The Effects of NAC on Oxidative Stress, Glutathione Concentration, Exploratory Behavior and Gait following MCAO

To assess the effects of NAC on oxidative stress, glutathione concentration and mobility following MCAO, 20 animals were assigned randomly to one of four main experimental groups: sham surgery with saline administration (Sham-Sal, n=5), sham surgery with NAC administration (Sham-NAC, n=5), MCAO surgery with saline administration (MCAO-Sal, n=5) and MCAO surgery with NAC administration (MCAO-NAC, n=5). Baseline measures of exploratory behavior and gait were taken one day prior to surgery and reassessed two days post-surgery. Approximately 30 minutes after the exploratory behavior was assessed, animals were euthanized and tissue was collected for the lipid peroxidation and glutathione peroxidase assays. Serum samples were also collected for the determination of circulating glutathione peroxidase.

Study 2: The Effects of NAC on Infarct Size and Gait following MCAO

To assess the effects of NAC on stroke volume and gait following MCAO, 19 mice were randomly assigned to one of two experimental groups: 1) MCAO surgery with saline administration (MCAO-Sal, n=9); 2) MCAO surgery with NAC administration (MCAO-NAC, n=10). Baseline measures of gait were taken one day prior to surgery and reassessed two days post-surgery. On post-operative day two, approximately 30 minutes after gait was assessed, animals were euthanized and

tissue was collected. Sectioned tissue was then stained using TTC in order to perform the stroke volume analysis.

Drug Administration

All animals were treated with two intraperitoneal injections of NAC (150mg/kg) or the saline vehicle. Drug administration occurred immediately prior to MCAO and again 24 hours following MCAO.

Surgery

The MCAO and sham surgeries were performed on anesthetized mice (isofluorane), using sterile surgical technique. Transient focal cerebral ischemia was induced under anesthesia by middle cerebral artery occlusion (MCAO). Briefly, unilateral MCAO was achieved by insertion of a 6-0 nylon monofilament into the internal carotid artery to a point 6mm beyond the internal carotid-pterygopalatine artery bifurcation. Once secured, the wound was sutured and the animal was allowed to awaken from anesthesia. After 60 minutes of occlusion, the animal was re-anesthetized and reperfusion was initiated by removal of the filament. For SHAM surgeries, the internal carotid artery was exposed but not disrupted.

Behavioral Testing

Each animal underwent baseline behavioral testing 24 hours prior to MCAO and again 48 hours after reperfusion. Behavioral testing was conducted during the light phase. The apparatuses were thoroughly cleaned between animals using a 70% alcohol solution.

Open Field

Exploratory behavior was assessed in an open field apparatus using Flex Field photobeam activity (San Diego Instruments, San Diego, CA). The apparatus was enclosed in a sound

attenuating chamber equipped with a ventilating fan which provides masking noise. A clear Plexiglas insert (40 X 40 X 37.5 cm) was fitted inside a metal frame consisting of 16 equally spaced infrared photocell detectors. Interruptions in the infrared light sources by the experimental animal were recorded in the associated computer program. Animals were individually placed inside the apparatus for 60-minute sessions and data were analyzed to determine general locomotor activity and relative amount of activity occurring in the periphery versus the center of the apparatus.

Gait Analysis

Assessment of gait dynamics and posture was assessed using DigiGait Imaging System (Mouse Specifics Inc., Quincy, MA). Animals were placed on a transparent treadmill and required to run while a camera below recorded the locomotor activity. 3-5 second segments of locomotor activity were used to analyze gait characteristics.

Glutathione Peroxidase Assay

Forebrain tissue was removed, homogenized in 20mM tris-buffered saline, and then centrifuged to remove insoluble components. Glutathione peroxidase activity was detected in the tissue homogenate with a specific assay kit (Cayman Chemical, Ann Arbor, MI). The kit measures glutathione peroxidase activity indirectly by a coupled reaction with recycled glutathione reductase. When glutathione peroxidase reduces hydroperoxide and produces oxidized glutathione, the glutathione reductase and NADPH can then recycle the glutathione to its reduced state. When NADPH is oxidized to NADP+, the absorbance is reduced to 350nm. The assay was conducted in a 96-well plate and read on a microplate reader. Sample glutathione peroxidase activity was derived from a standard curve.

Lipid Peroxidation

Lipids were extracted from forebrain homogenates with chloroform. Lipid peroxidation was assessed using a kit (Calbiochem). The kit detects lipid hydroperoxides directly. Hydroperoxides catalyze the conversion of ferrous ions into ferric ions. The resulting ferric ions are then detected with a thiocyanate-based chromagen. All samples for a single experiment were run in one assay and compared to a standard curve.

Infarct Size Analysis

Immediately following cervical dislocation and decapitation, fresh brains were removed, flash frozen and sectioned into five 2-mm-thick coronal section. They were then incubated for 12-minutes with 2,3,5-triphenyltetrazolium (TTC) at 37°C. Slices were post-fixed with 10% buffered formalin for three to six days before image analysis, at which point the slices were photographed and infarct area throughout the cerebrum will be analyzed using Inquiry software (Loats Associates, Inc., Rockville, MD). Infarct size was determined as a percentage of the contra-lateral hemisphere after correcting for edema.

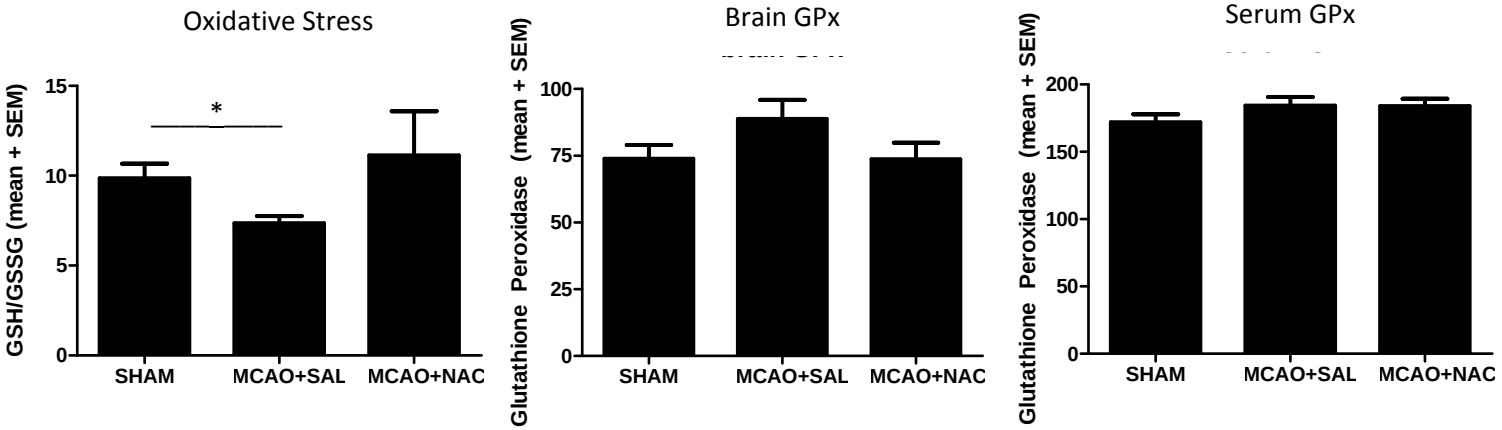
Statistical Analysis

The data are expressed as mean ± standard error of the mean. Testing of statistical significance was performed, using analysis of variance (ANOVA) or t-tests. When a significant overall treatment effect was reported, post hoc analyses were conducted, using the Tukey test. Group differences were considered statistically significant at $p<0.05$.

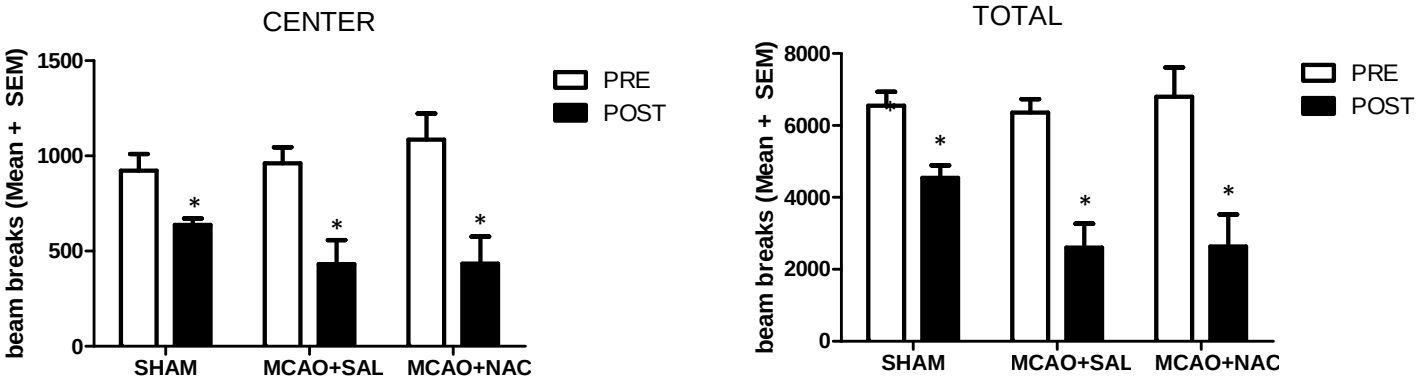
Results

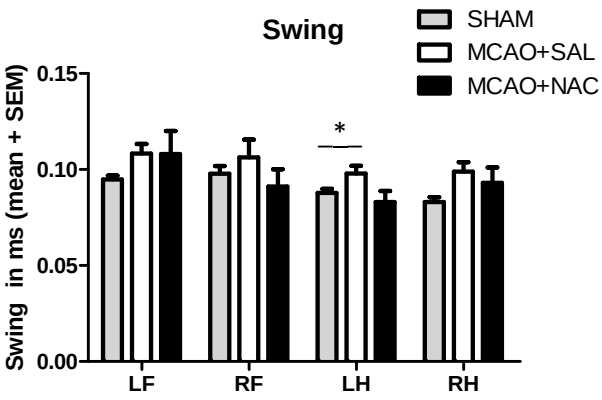
Study 1. NAC Reduces Oxidative Stress Gait Deficits to Sham Levels

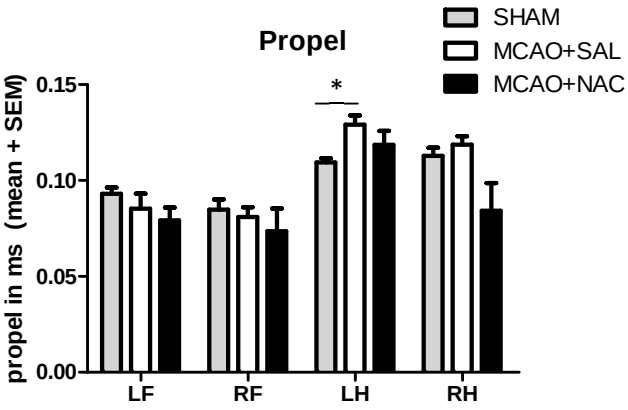
Among saline-treated animals, following MCAO, oxidative stress was significantly increased when compared with sham animals (SHAM vs. MCAO+SAL: $t_{18} = 2.74$, $p < 0.02$). However, NAC-treated mice that sustained MCAO displayed reduced levels of oxidative stress similar to those of sham mice. The assay for oxidative stress measures a ratio of ferrous ions to ferric ions. The higher the ratio, the lower the oxidative stress. Contrary to expectations, there were no significant differences in the brain or serum glutathione peroxidase enzyme concentrations among any of the groups. NAC treatment did not influence glutathione peroxidase concentration.

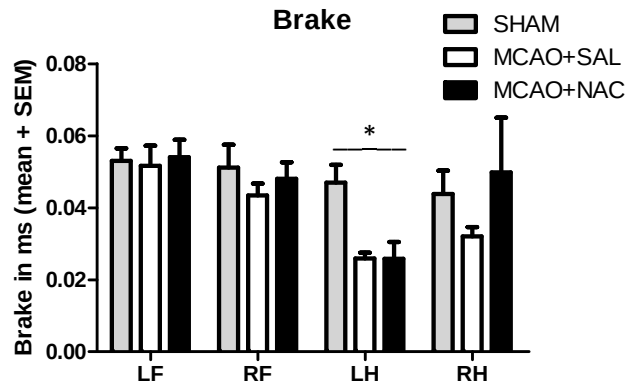


MCAO decreases exploratory behavior. Both sham groups exhibited similar levels of general locomotor activity and anxiety-like behavior in an open-field apparatus. Following MCAO, both saline and NAC-treated animals displayed a significant decrease in exploratory behavior (PRE vs. POST: Center - $t_{38} = 5.95$, $p < 0.01$; Total - PRE vs. POST: $t_{38} = 4.853$, $p < 0.01$).



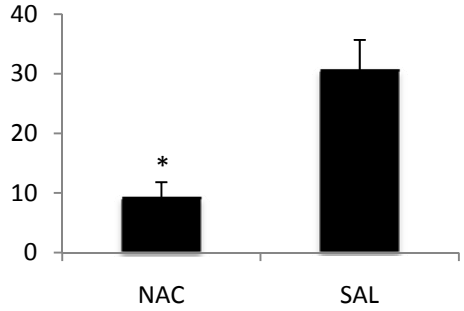






Gait analysis indicates that following MCAO, saline-treated mice show a significant increase in duration of swing in their affected hind leg when compared to sham animals (SHAM vs. MCAO+SAL: $t_{10} = 2.47$, $p < 0.05$). Although NAC-treated animals did not show a significant decrease in mean duration of swing compared to the saline-treated animals following MCAO, there was no difference between the NAC-treated animals and the sham animals. A similar pattern was apparent for the mean duration of propulsion. Following MCAO, saline-treated mice show a significant increase in mean duration of propulsion in their affected hind leg when compared to sham animals (SHAM vs. MCAO+SAL: $t_{10} = 3.26$, $p < 0.01$). Although NAC-treated animals did not show a significant decrease in mean duration of propulsion compared to the saline-treated animals following MCAO, there was no difference between the NAC-treated animals and the sham animals. Both saline-treated and NAC-treated animals showed a significant decrease in mean duration of braking following MCAO SHAM vs. MCAO = $t_{15} = 4.99$, $p < 0.01$).

Study 2: NAC Treatment Significantly Reduces Volume of Post-Ischemic Lesion



Following MCAO, NAC-treated animals showed a significant decrease in size

of infarct when compared with saline-treated animals (MCAO Saline vs. MCAO NAC = $t_{11} = 3.41$, $p < 0.01$).

Discussion

The increased production of ROS following cerebral ischemia and particularly during reperfusion is implicated in overwhelming the endogenous antioxidant systems leading to increased tissue damage. Evaluation of endogenous antioxidant systems or oxidized molecules in the brain tissue shows oxidative stress after ischemia. This oxidative stress is defined by decreased concentration of reduced glutathione.[8] The current study suggests that antioxidant treatment, specifically NAC, may increase the concentration of reduced glutathione, thus decreasing oxidative stress following MCAO. Although saline-treated mice show a significant increase in oxidative stress following MCAO, NAC-treated mice reduced oxidative stress so that there was no significant difference between NAC-treated mice and sham mice. Also, NAC treatment did not influence oxidative stress in the absence of the MCAO. Levels of glutathione peroxidase were not elevated in the brain or the serum of the animals following NAC treatment. This could be attributed to an already sufficient level of the enzyme in the body in order to catalyze the oxidizing reaction.

As expected, the MCAO procedure precipitated decreases in exploratory behavior assessed by open-field analysis. Following MCAO, both saline and NAC-treated groups decreased beam brakes in the center of the field, and overall this indicates a decrease in exploratory behavior. NAC treatment did not influence exploratory behavior. Furthermore, sham groups decreased exploratory behavior; however, this is likely attributed to a habituation response. Both saline and NAC-treated groups following MCAO experienced a much greater reduction in exploratory behavior than sham operated mice, indicating stroke-induced locomotor deficits.

Data trends suggest that NAC treatment following MCAO may improve gait. Mean swing duration is significantly increased in saline-treated mice following MCAO when compared to sham animals; however, mean swing duration is not significantly increased in NAC-treated mice following MCAO. Increased swing duration indicates decreased mobility. Mean propulsion duration is significantly increased in saline-treated mice following MCAO when compared to sham animals; however, mean propulsion duration is not significantly increased in NAC-treated mice following MCAO. An increase in propulsion duration suggests a decrease in strength and control in the affected limb. Mean brake duration was significantly decreased in both saline and NAC-treated animals when compared to sham animals. A decrease in brake duration indicates a decrease in precision of control and distribution of loading during stance. Taken together, these data provide the first evidence that treatment with NAC following stroke may improve mobility, thus indicating a neuroprotective effect from NAC.

The data from Study 2 replicated previous findings that NAC significantly influences the volume of the post-ischemic lesion. The volume of the post-ischemic lesion determined using a mitochondrial stain. The presence of mitochondria indicates the presence of living cells, whereas the absence of mitochondria indicates the presence of dead cells. Therefore, treatment with NAC prevents neuronal death. The concomitant decrease in oxidative stress and size of infarct, paired with the decrease in gait impairment in NAC-treated animals following MCAO supports the hypothesis that NAC treatment may work as a neuroprotective buffer against the damage caused by stroke. Reducing gait impairment in humans following stroke is particularly important because impaired gait function not only reduces ambulation, but could also lead to imbalance and falls. This is especially of concern in elderly patients experiencing stroke.[12] Numerous studies have recognized antioxidants as potential treatments for stroke.[1,8,11] No neuroprotective approaches, however, have been approved in the United States or Europe despite the success of a variety of compounds in

animal models of stroke.[8] Apart from the data in the present study, the antioxidant properties of NAC have been well described. Thus, in conjunction with previous reports on the effects of NAC and similar antioxidants on oxidative stress and infarct size, these data shed light onto the neuroprotective effects of NAC. The most important difference, however, between previous studies and the present work concerns the effects of NAC on gait impairment.

Acknowledgments

I would like to thank Dr. DeVries for hosting this research, and Dr. Karelina who oversaw and assisted with data collection. I would also like to thank Dr. Zhang who performed all surgeries and The Ohio State University Colleges of Arts and Sciences and Social and Behavioral Sciences for assisting with funding for this project.

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